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**HAIR FOLLICLE BULB AS A BIODOSIMETER  
FOR LOW-LEVEL VX VAPOR EXPOSURE:  
INITIAL STUDIES VALIDATING THE PRESENCE  
OF POTENTIAL PROTEIN BIOMARKERS OF EXPOSURE  
IN THE SPRAGUE-DAWLEY RAT WHISKER FOLLICLE**

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**October 2006**

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14. ABSTRACT Over the past fifty years, numerous studies have been performed to determine the health effects of exposure to organophosphorus (OP) nerve agents. The traditional approaches to determine OP exposure are invasive (e.g. require blood samples) laboratory-based assays that are not ideal for rapid, reliable testing in the event of a mass exposure scenario. However, advances in biomarker discovery make it possible to develop a less invasive, more expedient assay. The active hair follicle bulb expresses a large number of proteins, is easily accessible, and is an excellent candidate from which to assess biomarkers. In this report, we describe a novel immunohistochemistry (IHC) method developed for this initial hair follicle investigation, as well as the initial work completed to determine the presence of fifteen potential protein OP agent exposure biomarkers in the rat whisker follicle bulb. Each potential biomarker protein was chosen because of its change in expression in non-hair follicle tissue following exposure to pesticides and/or OP nerve agents. Using Western blotting, the expression of these proteins was verified in the rat whisker follicle bulb. Following verification in the digested hair follicle, intact whole-hair IHC was performed to determine protein expression levels in the intact whisker follicle bulb.													
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## PREFACE

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## CONTENTS

1.	INTRODUCTION .....	7
2.	MATERIALS AND METHODS.....	8
2.1	Collection of Rat Whiskers.....	8
2.2	Specific Protein Detection .....	8
2.2.1	Antibodies.....	8
2.2.2	SDS-PAGE and Western Blotting of Hair Protein .....	10
2.3	Immunohistochemistry .....	12
3.	RESULTS .....	12
3.1	Western Blot Confirmation.....	12
3.2	Proteinase K Timecourse .....	13
3.3	Intact Hair Follicle Bulb IHC .....	14
4.	DISCUSSION .....	15
	LITERATURE CITED .....	19

## FIGURES

1.	Western Blot of Total Rat Follicle Bulb Protein Separated by 10-20% SDS-PAGE .....	13
2.	Proteinase K Digestion of the Rat Whisker Follicle Bulb .....	14
3.	IHC of Negative Controls and Confirmed Targets .....	15

## TABLES

1.	Biomarker Candidates in the Rat Whisker Follicle Bulb.....	9
2.	Heat Treatment of Hair Extract and Antibody Concentrations for Western Blot and IHC.....	11

# HAIR FOLLICLE BULB AS A BIODOSIMETER FOR LOW-LEVEL VX VAPOR EXPOSURE: INITIAL STUDIES VALIDATING THE PRESENCE OF POTENTIAL PROTEIN BIOMARKERS OF EXPOSURE IN THE SPRAGUE-DAWLEY RAT WHISKER FOLLICLE

## 1. INTRODUCTION

The skin covering the human body is rich in hair and hair follicles, with over 90% of its surface supporting some type of hair.<sup>1</sup> Biologically defined, hair is the epidermal tissue that is derived from the invaginations, called follicles, located in the skin.<sup>2</sup> The bulb region of the hair follicle contains the metabolically active cells of the hair, which derive nutrients from the circulating blood.<sup>3</sup> As such, hair follicle bulb cells are responsive in real-time or near real-time to toxic insults, in much the same way as skin cells. Chemicals or heavy metals present in the body are brought into contact with the hair follicle by the circulating blood. There, metals may enter the follicle and may potentially be incorporated into the hair shaft by the proliferating matrix cells found in the hair bulb.<sup>2</sup> The ability to be incorporated into the hair shaft has led to the possibility of detecting exposure to chemicals and heavy metals via the hair shaft. Termed segmental hair analysis, this has become a popular method to determine exposure to drugs of abuse (e.g. cocaine, amphetamine, methamphetamine, heroin, marijuana, and steroids), therapeutic drugs (e.g. ephedrine, benzodiazepines, and barbiturates), and inorganic heavy metals (e.g. arsenic, lead, and mercury).<sup>4-7</sup>

Of particular relevance to the work described in this manuscript are recent reports suggesting that long-term exposure to pesticides (e.g. carbamate, organochlorine, and organophosphate (OP)) can also be detected using segmental hair analysis. Tsatsakis and colleagues demonstrated that methomyl, a carbamate pesticide, could be detected in the rabbit hair shaft following a 4-month daily methomyl exposure. They noted that the amount of methomyl incorporated into the hair was dependent on the growth rate of the hair.<sup>8</sup> Later studies showed that organochlorine pesticides, too, could be detected in human hair. Specifically, PCB, DDT, and Lindane could all be detected in both natural and artificially colored hair.<sup>9</sup> Two later studies were performed in which chronic exposure to the OP pesticide diazinon was monitored using segmental hair analysis. In the first study, segmental hair analysis was used to assess chronic exposure to diazinon in white New Zealand rabbits exposed to high- (15 mg/kg, 0.1 LD<sub>50</sub>) and low-doses (8 mg/kg, 0.06 LD<sub>50</sub>) of diazinon via drinking water for 4 months. As expected, decreased serum acetylcholinesterase (AChE, EC 3.1.1.7) levels corresponded with increased concentrations of diazinon in the hair shaft.<sup>10</sup> The second study repeated the rabbit study protocol, but used Sprague-Dawley rats, a more pesticide resistant animal. Since rabbits are more efficient than rats in transforming diazinon into diazoxon, a decrease AChE occurs more quickly and at lower concentrations. In fact, the LD<sub>50</sub> for diazinon in rabbits is half that of the LD<sub>50</sub> of the Sprague-Dawley rat.<sup>11</sup> The rats were exposed to high- (6 mg/kg, 0.02 LD<sub>50</sub>) and low-doses (3 mg/kg, 0.01 LD<sub>50</sub>) of diazinon via drinking water for 1.5 months. As expected, the increase in the amount of pesticide in the drinking water directly correlated with an increase in the concentration of diazinon found in the hair.<sup>11</sup>

To carry out segmental hair analysis, scalp hair is cut into 1, 2, and 3 cm segments, corresponding to approximately 1, 2, and 3 month's growth. If scalp hair is not available, other sources of hair can be used for analysis. Segmental analysis can be accomplished with as little as one hair or up to 200 mg of hair (to allow confirmation testing).<sup>7</sup> The segments are typically washed to remove external contaminants and the chemicals in the hair are extracted by alkaline digestion, enzymatic treatment, or acid extraction. Several analytical methods are available to determine the concentration, if any, of a particular substance in the hair. Immunoassay, High Performance Liquid Chromatography (HPLC),

Capillary Electrophoresis (CE), Gas Chromatography-Mass Spectrometry (GC-MS), tandem mass spectrometry (MS-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS) can be used for analysis. Of these, the superior choice for high sensitivity, selectivity, and specificity is GC-MS separation and selective ion monitoring (SIM) quantitation. However, tandem MS can be used if the compound of interest is unstable in gas chromatograph separation/analysis.<sup>6</sup>

The main advantage of segmental hair analysis is that successful analysis is possible even months after exposure. Other sample sources for testing, such as urine, offer an immediate window of testing, however, most drugs can only be detected 1 to 3 days following initial use.<sup>12</sup> Although segmental hair analysis is becoming increasingly popular, the risk of a false positive is high when compared to urine or blood testing. Dark hair color, poor personal hygiene, and passive exposure can also lead to false positive segmental hair analysis results.<sup>13</sup>

Due to the increased risk of false positives associated with segmental hair analysis and the more invasive collection required for serum and urinalysis, work to develop a new method using the living, responsive cells in the plucked hair follicle bulb was initiated. The new method described in this manuscript relies upon detection of OP agent responsive proteins (biomarkers) in intact, plucked hair follicle bulbs using a novel immunohistochemistry (IHC) method. Rather than measuring the toxicant or its metabolites, changes in specific toxicant responsive follicle bulb proteins are monitored to determine toxicant exposure. Since there is an interest to determine OP nerve agent exposure, the pesticide and OP nerve agent open literature was mined and 17 potential biomarkers were chosen for this work (see Table 1). This manuscript describes the identification of these potential biomarkers in the Sprague-Dawley rat whisker bulb and the modifications to the classical IHC procedure used to measure them.

## 2. MATERIALS AND METHODS

### 2.1. Collection of Rat Whiskers.

Whiskers of male and female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were pulled manually, with care to remove intact whisker bulbs and immediately snap frozen in liquid nitrogen and stored at  $-135^{\circ}\text{C}$  until analysis.

### 2.2 Specific Protein Detection.

#### 2.2.1 Antibodies.

The mouse monoclonal antibodies used in this work were: anti-Actin (Novus Biologicals, Littleton, CO), anti-Breast Cancer Antigen 1 (BRCA 1) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Glial cell-line derived neurotrophic factor (GDNF) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Glutathion S-Transferase (GST) (Chemicon, Temecula, CA), anti-Keratin 10 (Neo Markers, Fremont, CA), anti- $\text{Na}^{+}/\text{K}^{+}\text{ATPase } \alpha 1$  (Santa Cruz Biotechnology, Santa Cruz, CA), and anti- $\text{Na}^{+}/\text{K}^{+}\text{ATPase } \beta 1$  (Santa Cruz Biotechnology, Santa Cruz, CA).



Table 1. Biomarker Candidates in the Rat Whisker Follicle Bulb.

Potential Biomarker	Function	Literature evidence (tissue type)
Actin	Cytoskeletal; cell division	Rats exposed to sublethal doses of soman reported to have decreased levels of protein synthesis (brain) <sup>14</sup>
Aldehyde Dehydrogenase (ALDH)	Metabolism; Phase II	Cells exposed to methylcholanthrene showed induction of ALDH (cell culture) <sup>15</sup>
Androgen Receptor	Hormone Receptor	OP and OC pesticides were shown to act as antagonists of the AR (cell culture) <sup>16</sup>
Brain derived neurotrophic factor (BDNF)	Neurotrophin	BDNF mRNA down-regulated in animals exposed to OP pesticides (brain) <sup>17</sup>
CD 3	Lymphocytes (T-lymphocytes)	Mice exposed to low-level sarin showed slight decreases of CD 3 (lungs) <sup>18,19</sup>
CD 20	Lymphocytes (B-lymphocytes)	Mice exposed to low-level sarin showed slight increases of CD 19 (lungs) <sup>18-19</sup>
Cytochrome P450 1A1 (CYP 1A1)	Metabolism; Phase I	Cells exposed to $\beta$ -naphthooflavone, phenobarbital and methylcholanthrene showed increase CYP1A1 activity (cell culture) <sup>15</sup>
Estrogen Receptor $\alpha$	Hormone Receptor	OP pesticide diazinon demonstrated estrogenic activity in ovarian carcinoma cells (cell culture) <sup>20</sup>
Glial cell line-derived neurotrophic factor (GDNF)	Neurotrophic factor; cytokine family	GDNF mRNA expression increased in rats exposed to sub-chronic doses of phencyclidine (rat brain); <sup>21</sup> also known to aid in repair following neuronal damage
Glucose Transporter	Membrane bound; transport	Cells exposed to lipophilic pesticides showed a decrease in glucose transporter activity (cell culture) <sup>22</sup>
Glutathion-S-Transferase (GST)	Metabolism; Phase II	Cells exposed to methylcholanthrene showed induction of GST (cell culture) <sup>15</sup>
NAD(P)H: Quinone Oxidoreductase I (NQO I)	Metabolism; Phase II	Cells exposed to methylcholanthrene showed induction of NQO I (cell culture) <sup>15</sup>
Sodium-Potassium ATPase ( $\alpha$ and $\beta$ sub-units)	Membrane bound; ion transport	Cells exposed to lipophilic pesticides showed a decrease in $\text{Na}^+/\text{K}^+$ -ATPase activity (cell culture) <sup>22, 23</sup>
$\alpha$ -Tubulin	Cytoskeletal; cell division	Rats exposed to sublethal doses of soman had decreased levels of protein synthesis (brain) <sup>14</sup>
$\beta$ -Tubulin	Cytoskeletal; cell division	Rats exposed to sublethal doses of soman had decreased levels of protein synthesis (brain) <sup>14</sup>
Tumor Necrosis Factor - $\alpha$ (TNF- $\alpha$ )	Cytokine	Rats exposed to low-level sarin shown increased mRNA for TNF- $\alpha$ (brain) <sup>24, 25</sup>

The rabbit polyclonal antibodies used in this work were: anti-Androgen Receptor (AR) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Brain Derived Neurotrophic Factor (BDNF) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Cytochrome P450 1A1 (CYP1A1) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD20 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Estrogen Receptor  $\alpha$  (ER  $\alpha$ ) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Glyceraldehyde Phosphate Dehydrogenase (GAPDH) (Novus Biologicals, Littleton, CO), anti-Glucose Transporter 1 (Glut 1) (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-NAD(P)H: quinone oxidoreductase I (NQO 1) (Santa Cruz Biotechnology, Santa Cruz, CA).

The goat polyclonal antibodies used in this work were: anti-Aldehyde Dehydrogenase 1A2 (ALDH1A2) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Tissue Non-Specific Alkaline Phosphatase (TNAP) (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) (Santa Cruz Biotechnology, Santa Cruz, CA).

The chicken polyclonal antibody used in this work was: anti-Matrix Metalloproteinase-2 (MMP-2) (Abcam, Cambridge, MA).

The Cy5-labeled goat anti-rabbit IgG and Cy5-labeled goat anti-mouse IgG were purchased from Amersham Biosciences (Pisataway, NJ). Cy5-labeled and Cy3-labeled rabbit anti-goat IgG was purchased from Chemicon (Temecula, CA). Alexa Fluor 532-labeled anti-Chicken IgY was made from rabbit anti-Chicken IgY (Abcam, Cambridge, MA) labeled with the Alexa Fluor 532 Protein Labeling Kit (Molecular Probes, Eugene, OR). Alkaline phosphatase conjugated anti-rabbit IgG, anti-mouse IgG, anti-goat IgG and anti-chicken IgY were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

#### 2.2.2 SDS-PAGE and Western Blotting of Hair Protein.

Protein was extracted from the bulb region of 15 to 25 hairs from donor rats. The hairs were cut to separate the bulb and shaft regions. The bulb region was then incubated in buffer as described by Inoue et al. at 37 °C for 96 hr.<sup>26</sup> Following extraction, the proteins were dialyzed against 0.2 M Tris-HCl, pH 8.0 containing Complete Protease Inhibitor Cocktail tablets (Roche, Indianapolis, IN) at room temperature for 4 hr (2 buffer changes) and then at 4 °C overnight. Protein concentration was determined using the DC Protein Assay kit (BioRad, Hercules, CA). Extracts were separated by SDS-PAGE using a 10-20% Tris-HCl Ready Gel (BioRad, Hercules, CA). Equal amounts of the hair protein extract were heated in an equal volume of 2X Sample Buffer (62.5 mM Tris-HCl, pH 6.8 containing 2% SDS, 20% Glycerol, and 0.01% Bromophenol Blue) at the temperature and time indicated in Table 2. Kaleidoscope size markers (BioRad, Hercules, CA) were run concurrently in each gel.

Following electrophoresis, the gel was incubated for 15 min in Transfer Buffer (25 mM  $K_2HPO_4$ , 25 mM  $KH_2PO_4$ , and 0.25 mM EDTA). The proteins were then transferred onto 0.45  $\mu$ m nitrocellulose membranes (Schleicher and Schuell, Keene, NH) in a BioRad Mini Protean Transfer Cell (BioRad, Hercules, CA).

Table 2. Heat Treatment of Hair Extract and Antibody Concentrations for Western Blot and IHC.

Potential Protein Biomarker	Heat Treatment of Hair Extract	Primary Antibody Origin	Concentration of Primary Ab for Western Blot	Concentration of Secondary Ab for Western Blot	Concentration of Primary Ab for IHC
Actin	3 min (100 °C)	Mouse	1:75	1:5000	1:25
ALDH 1A2	30 min (65 °C)	Goat	1:30	1:28000	1:25
AR	3 min (100 °C)	Rabbit	1:25	1:12000	1:25
BDNF	3 min (100 °C)	Rabbit	1:25	1:18000	1:25
BRCA 1	3 min (100 °C)	Mouse	1:125	1:10000	1:25
CD 3	10 min (95 °C)	Mouse	1:30	1:20000	N/A
CD 20	3 min (100 °C)	Rabbit	1:75	1:16000	1:25
CYP 1A1	3 min (100 °C)	Rabbit	1:100	1:6000	1:25
ER $\alpha$	30 min (65 °C)	Rabbit	1:20	1:28000	1:25
GAPDH	3 min (100 °C)	Rabbit	1:150	1:6000	1:25
GDNF	3 min (100 °C)	Mouse	1:25	1:2500	1:25
Glut 1	3 min (100 °C)	Rabbit	1:100	1:6000	1:25
GST	30 min (65 °C)	Mouse	1:75	1:12000	1:25
Keratin 10	3 min (100 °C)	Mouse	1:100	1:2000	1:25
MMP-2	30 min (65 °C)	Chicken	1:500	1:15000	1:50
NQO 1	30 min (65 °C)	Rabbit	1:30	1:28000	1:25
Na <sup>+</sup> /K <sup>+</sup> -ATPase $\alpha$ 1	30 min (65 °C)	Mouse	1:20	1:12000	N/A
Na <sup>+</sup> /K <sup>+</sup> -ATPase $\beta$ 1	3 min (100 °C)	Mouse	1:25	1:2500	1:25
TNF- $\alpha$	30 min (65 °C)	Goat	1:30	1:28000	1:25
TNAP	10 min (95 °C)	Goat	1:20	1:23000	1:25
$\alpha$ -Tubulin	3 min (100 °C)	Mouse	1:100	1:3000	1:25
$\beta$ -Tubulin	30 min (65 °C)	Mouse	1:50	1:12000	1:25

Following transfer, the nitrocellulose membranes were incubated with Antibody Extender Solution (Pierce, Rockford, IL) at room temperature for 10 min. The nitrocellulose membranes were blocked in TTBS (50 mM Tris, pH 8.0 containing 150 mM NaCl and 0.5% Tween) containing 5% BSA overnight at 4 °C. Primary antibodies were diluted to a concentration of between 1:20 and 1:500 in TTBS containing 5% BSA (see Table 2 for dilutions). The membranes were incubated with the primary antibody at 37 °C for 4 hr. The membranes were washed with TTBS 4 times, 5 min per wash. Dye-conjugated secondary antibodies were diluted in TTBS containing 5% BSA (see Table 2 for dilutions). The membranes were incubated in secondary antibody at room temperature for 1 hr. Following removal from secondary antibody, the membranes were washed in TTBS 4 times, 5 min per wash, and rinsed in diH<sub>2</sub>O 3 times, 2 min per rinse. Membranes were developed with NBT/BCIP (Roche, Indianapolis, IN) at room temperature until sufficient color development occurred.

### 2.3 Immunohistochemistry.

Hair samples were fixed briefly in fresh acetone, removed and incubated in TBS (50 mM Tris, pH 8.0 containing 150 mM NaCl) at room temperature for 15 minutes. The hairs were mounted onto glass slides, leaving the bulb and lower shaft of the follicle free and digested with 0.1 mg/ml Proteinase K (Invitrogen, Carlsbad, CA) in 20 mM Tris-HCl, pH 7.5 containing 50 mM CaCl<sub>2</sub> at 37 °C for 1 hr. Following digestion, the entire slide was washed with TTBS 4 times, 5 min per wash. Individual hair follicle lower shaft and bulb were blocked at room temperature for 10 minutes with TTBS containing 5% BSA. The blocking solution was blotted off and each hair follicle lower shaft and bulb was covered with primary antibody (1:25-1:50) (see Table 2 for dilutions). Slides were incubated at 4 °C overnight in a humidity chamber. Following incubation with the primary antibody, slides were washed with TTBS 4 times, 5 min per wash. Each lower shaft and hair follicle bulb was immersed in Cy5-labeled secondary antibody (1:400). Slides were incubated at room temperature for 1 hr in darkness. Following secondary antibody incubation, slides were washed 4 times, 5 min per wash, with TTBS. For triple labeling, 10 µl of an anti-TNAP (1:25) and anti-MMP-2 (1:50) mixture was used to cover each hair follicle lower shaft and bulb. Slides were incubated in a humidity chamber at room temperature for 1 hr. Following incubation with the anti-TNAP/anti-MMP-2 mixture, the slides were washed with TTBS 4 times, 5 min per wash. Hairs were covered with a mixture Cy3-labeled rabbit anti-goat IgG (1:400) and Alexa 532 rabbit anti-chicken IgY (1:400) in a dark humidity chamber at room temperature for 1 hr. Slides were washed with TTBS 4 times, 5 min per wash, and rinsed with diH<sub>2</sub>O 3 times, 2 min per wash. Images were analyzed on Affymetrix 428 scanner (Affymetrix, Santa Clara, CA) using Affymetrix Jaguar 2.0 software (Affymetrix, Santa Clara, CA).

## 3. RESULTS

### 3.1 Western Blot Confirmation.

All targets chosen for this study were due to their role in skin biology or OP pesticide or nerve agent response. Western Blots were performed to ensure that these proteins of interest were located in the hair bulb. Figure 1 shows Western blotting confirmation of all targets. Of the 17 potential targets, all could be verified in the bulb region of the follicle except CD3 and Na<sup>+</sup>/K<sup>+</sup>-ATPase α1.

CD 3 is the designation given to the multi-subunit complex of proteins that associate with the T cell antigen receptor (TCR). Christoph et al. report that only 5 cells out of 100 hair follicles express TCR in the distal outer root sheath, thus TCR is found extremely rarely in the hair follicle.<sup>27</sup> The reported rarity of finding TCR in the hair follicle could explain why the Western blotting carried out in this work did not confirm CD 3 in the rat whisker hair follicle bulb.

The Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$ 1 subunit was the other potential target that could not be confirmed by the Western blotting in our experiments. However, the Western blotting confirmed the  $\beta$ 1 subunit of the Na<sup>+</sup>/K<sup>+</sup>-ATPase among follicle proteins. The  $\alpha$ 1 and  $\beta$ 1 subunits are found in a 1:1 ratio in the plasma membrane complex Na<sup>+</sup>/K<sup>+</sup>-ATPase.<sup>28</sup> The lack of confirmation of the  $\alpha$ 1 subunit might be due to the epitope of the antibody used or the fact that 32% of the  $\alpha$  subunit protein mass is found in the lipid-associated membrane region. Only a small portion of the  $\beta$ -subunit protein mass is found in the lipid associated region, which could explain the confirmation of the  $\beta$ -subunit in the rat whisker follicle by Western blotting.<sup>29</sup>

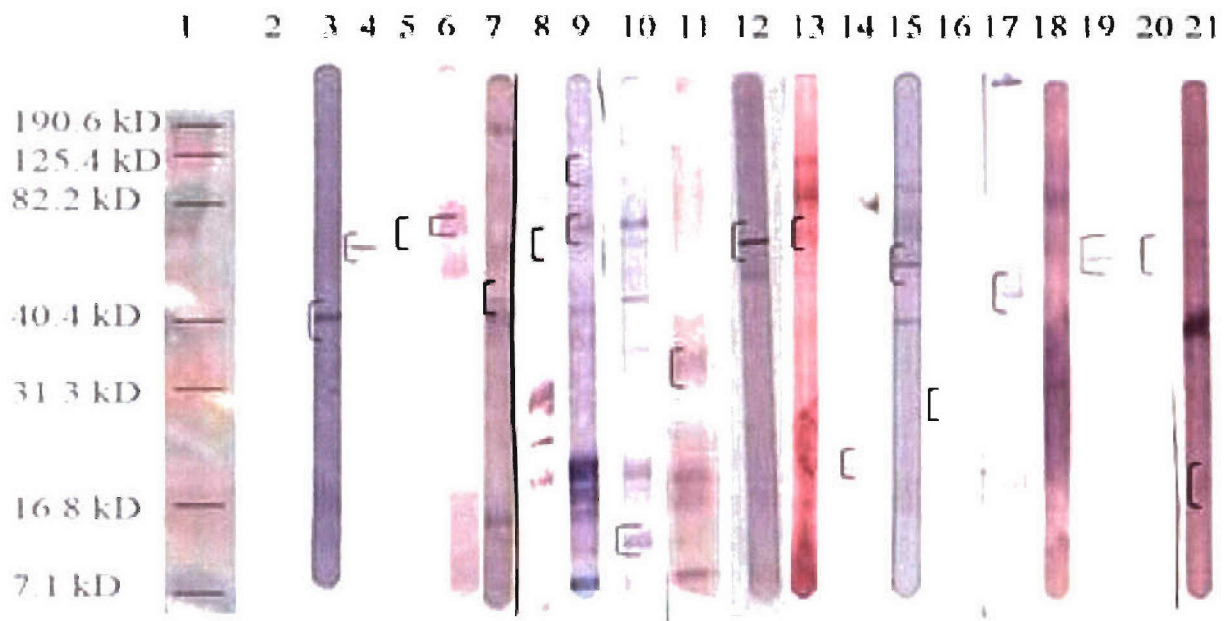


Figure 1. Western Blot of Total Rat Follicle Bulb Protein Separated by 10-20% SDS-PAGE. The lanes are 1: BioRad Kaleidoscope Molecular Weight Ladder; 2: BRCA 1; 3: GAPDH; 4: Keratin 10; 5: MMP-2; 6: TNAP; 7: Actin; 8: ALDH 1A2; 9: AR; 10: BDNF; 11: CD 20; 12: CYP 1A1; 13: ER  $\alpha$ ; 14: GDNF; 15: GLUT 1; 16: GST; 17: Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\beta$ 1; 18: NQO 1; 19:  $\alpha$ -Tubulin; 20:  $\beta$ -Tubulin; 21: TNF- $\alpha$ .

### 3.2 Proteinase K Timecourse.

Proteinase K (EC 3.4.21.14) is a non-specific serine protease commonly used in IHC to digest tissue samples, to retrieve epitopes, and to reduce background immunoreactivity. Termed Protease-induced epitope retrieval (PIER), this technique was first described in 1975 by Huang et al. for the retrieval of keratins in cancerous tissue.<sup>30</sup> In this technique, enzymes such as trypsin, proteinase K, pronase, ficin, and/or pepsin are used to non-specifically digest proteins in a fixed sample in order to retrieve epitopes of interest.<sup>31</sup> However, care must be taken in order to ensure that epitopes are not destroyed by excessive enzyme digestion.



The PIER technique employed for this work was achieved by Proteinase K in order to digest the fibrous Outer Root Sheath (ORS) of the rat whisker follicle bulb. To optimize this technique for the investigation of the rat whisker bulb, a series of PIER time course experiments were carried out. The efficiency of the Proteinase K digestion was measured using anti-Keratin 10 and anti-MMP-2. Keratin 10, a positive control protein, is expressed in the epidermis and inner-root sheath (IRS) of normal human and dog hair.<sup>32</sup> However, Keratin 10 is not expressed in the ORS. In order to control for the growth stage of the whisker, the protein MMP-2 was probed. However, unlike Keratin 10, it is expressed in the ORS and other layers within the hair bulb.<sup>33,34</sup>

By using the growth stage indicator, MMP-2, expressed in the ORS, the growth stage of the hair follicle bulb was matched between whiskers used in this PIER optimization. It was expected that when the ORS was still intact, there would be no interaction of the Keratin 10 antibody with the protein Keratin. Only after the Proteinase K digestion of the ORS, was Keratin 10 immunoreactivity expected to be observed. The Proteinase K digestion optimization was aimed toward exposing the proteins found beneath the ORS.

As expected, there was minimal Keratin 10 immunoreactivity (Figure 2a), but strong MMP-2 immunoreactivity (data not shown) before any Proteinase K digestion. Following 30 and 60 minutes of digestion, the ORS was digested, as evidenced by the abundance of Keratin 10 antibody binding (Figure 2b and 2c). As the ORS was digested, MMP-2 was still clearly observed in the IRS. One of the dangers of PIER is the possibility of over-digestion, resulting in destruction of the epitope of interest. As shown in Figure 2d, after 90 minutes of Proteinase K digestion, some of the Keratin 10 epitope had been destroyed. This is evidenced by the little relative Keratin 10 immunoreactivity. Based on the data shown in Figure 2, a 60-minute digestion was determined optimal for these given PIER conditions, as the ORS was digested, yet the epitopes containing the regions of interest were not damaged.

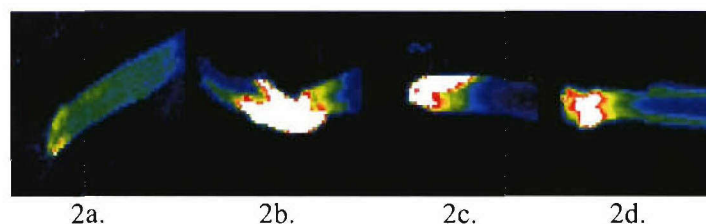


Figure 2. Proteinase K Digestion of the Rat Whisker Follicle Bulb. Figure 2a shows an undigested whisker bulb, Figure 2b-d show whisker bulbs digested with Proteinase K for 30 min (b), 60 min (c), and 90 min (d).

### 3.3 Intact Hair Follicle Bulb IHC.

In order to further confirm the presence of potential targets in the hair bulb, IHC using antibodies for the specific target proteins was performed (Figure 3). All proteins with positive results in Western blotting experiments were further verified in the intact hair follicle bulb using modified, whole mount IHC.

In order to ensure that observed immunoreactivity results were not due to non-specific binding of the secondary antibody, negative controls experiments were performed. The chicken, goat,



and rabbit secondary antibodies used in the IHC all produce minimal background immunoreactivity (Figure 3b-c and 3e). However, using a mouse secondary antibody results in slightly higher background levels (Figure 3d). This increased background observed when using a mouse secondary antibody is likely the result of the significant protein homology between the rat and mouse. Based on these results, it was determined that polyclonal antibodies produced in chicken, goat, and rabbit will be used.

An additional negative control antibody, anti-BRCA 1, was employed in these experiments. Since BRCA 1 proteins are not found in the hair follicle, no fluorescence should be observed. As shown in Figure 3a, the total fluorescence for the hair bulb stained for BRCA 1 is negligible. The hair bulb probed with anti-BRCA 1 in this example appears to have less background than the hair bulb probed with only the mouse secondary antibody. While both bulbs in this example were actively growing, analysis of the MMP-2 and TNAP immunoreactivity patterns reveals they are not in the same growth stage. If the bulb of the hair probed with anti-BRCA 1 was in the same growth stage as the mouse secondary antibody negative control, the background levels would be similar.

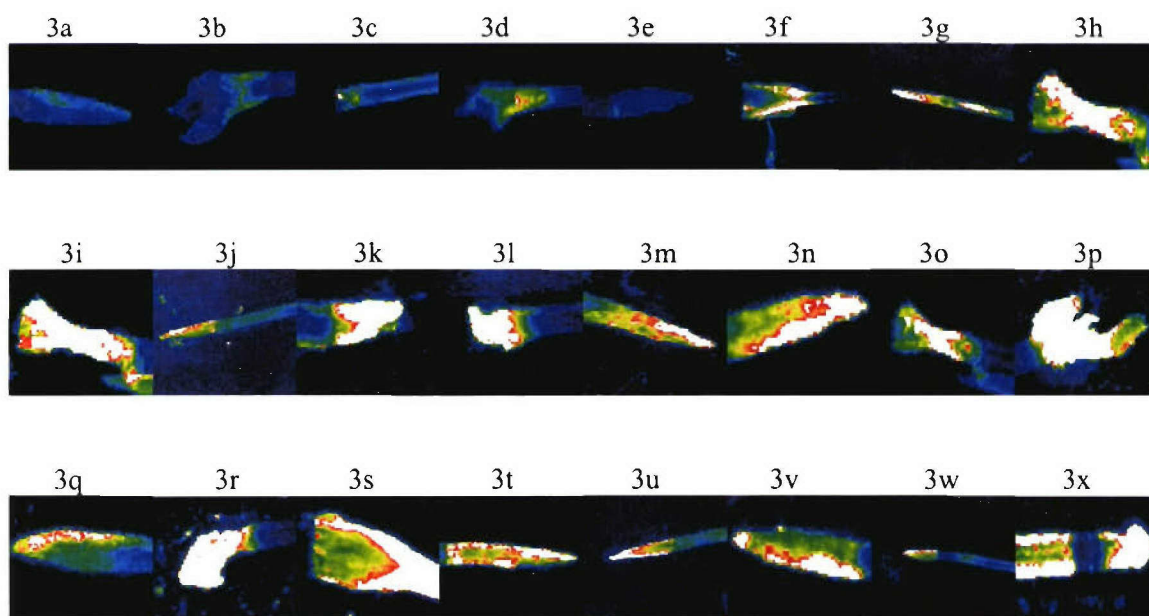


Figure 3. IHC of Negative Controls and Confirmed Targets. Fluorescence scanning images of rat whisker follicle bulbs. The figures are as follows: 3a: BRCA 1; 3b: Chicken secondary negative control; 3c: Goat secondary negative control; 3d: Mouse secondary negative control; 3e: Rabbit secondary negative control; 3f: GAPDH; 3g: Keratin 10; 3h: MMP-2; 3i: TNAP; 3j: Actin; 3k: ALDH 1A2; 3l: AR; 3m: BDNF; 3n: CD 20; 3o: CYP 1A1; 3p: ER  $\alpha$ ; 3q: GDNF; 3r: Glut 1; 3s: GST; 3t: NQO 1; 3u:  $\text{Na}^+/\text{K}^+$ -ATPase  $\beta$ 1; 3v:  $\alpha$ -Tubulin; 3w:  $\beta$ -Tubulin; and 3x: TNF- $\alpha$ .

#### 4. DISCUSSION

Carbamate, OP pesticides and OP nerve agents exert their effects through the inhibition of AChE. Acetylcholinesterase is the enzyme responsible for the breakdown of the neurotransmitter acetylcholine (ACh). Following AChE inhibition, ACh accumulates in the synapses. As a result of the increased ACh concentration in the synapses, the portions of the sympathetic and parasympathetic nervous system that control smooth muscle, cardiac muscle and exocrine glandular function are overstimulated.<sup>35</sup> Urine and blood tests are available to determine the presence of OP compound

hydrolysis products. Although conclusive, most require derivatization prior to analysis.<sup>36</sup> Currently, measurement of AChE levels in the blood is the fastest and most commonly employed method to determine exposure to pesticides and nerve agents. Although this test is faster than analyzing for hydrolysis products in the urine or blood, there are still several drawbacks to the test due to the inherent nature of AChE. First, there is a 10-18% interindividual variation and a 3 to 7% intraindividual variation for AChE levels.<sup>37</sup> Thus, without a known personal baseline, it is difficult to conclude if a person has been exposed to a nerve agent if they show inhibition levels of less than 20%. Secondly, due to the *de novo* synthesis of new AChE, this method cannot be used for retrospective determination of exposure.<sup>36</sup> Thus, a need exists to develop a new simple, rapid method to determine exposure to carbamate and OP pesticides and OP nerve agents.

In order to provide an alternative to invasive tests using blood or urine, a new methodology using plucked hair follicle bulbs was developed. Plucked hair follicle bulb collection is relatively non-invasive, can be performed in the field, and samples can be easily preserved, stored, and shipped. Additionally, enough hair samples can easily be collected and stored in order to repeat testing for further confirmation. The method overcomes the false positives associated with traditional segmental hair analysis such. By measuring the changes in specific biomarker levels in the living portion of the hair follicle bulb, the IHC technique employed in this method detects physiological response to exposure in near real-time.

The concept of IHC began in the 1930s when several studies reported that antibodies could be “marked” with dye-groups and still retain their specific immunoreactivity.<sup>38,39</sup> Several years later, Marrack demonstrated with labeled anti-typhoid serum and unlabeled anti-cholera serum that the color seen in the agglutinated typhoid was due to specific antibody binding and not non-specific absorption or occlusion of the dye in the cell.<sup>40</sup> However, Coons and colleagues are credited with describing the first IHC study with fluorescently labeled pneumococcal antigens on mouse tissue sections. They describe that the immunofluorescence of mouse tissue infected with pneumococcus was observed in localized areas and demonstrated that the staining was immunologically specific.<sup>41</sup>

Coons and colleagues successfully bridged the fields of immunology, chemistry, and histology, resulting in a simple, yet powerful research and diagnostic tool. Antibodies are used to identify specific antigens within tissue sections. Following antigen-antibody binding, the antigen is then demonstrated in the tissue with a colored dye or fluorochrome.<sup>31</sup> Because intact tissue is used, IHC can be used to determine the location of specific antigens. In the more than six decades since Coons and colleagues published their study, many improvements have been made in IHC, allowing it to become a commonly employed laboratory technique.

While hair has been used as a reservoir for markers of other types of exposures, such as to heavy metals and both therapeutic drugs and drugs of abuse, very little work has employed IHC on intact hair follicle bulbs to investigate changes in the living cells of the hair.

Hair follicles cycle through three stages: growth (anagen), involution (catagen) and rest (telogen).<sup>42</sup> A number of proteins cycle along with the hair follicle growth stages, thus the growth stage of individual hairs can be assessed by the presence or absence of these specific cycling proteins. Since hair follicle bulbs must be growth stage matched in order to compare levels of proteins that change in response to exposures of interest, accurate assessment of growth stage is extremely important. The two control proteins used in combination in this work, alkaline phosphatase (AP) and matrix metalloproteinase-2 (MMP-2), serve this function very well.

Alkaline Phosphatase (EC 3.1.3.1) is a zinc metalloenzyme with a wide range of cellular functions. There are four distinct forms of AP: Intestinal (IAP), Placental (PLAP), Placental-like (ALP-1) and Tissue Non-Specific (TNAP).<sup>43</sup> The Tissue Non-Specific isoform of Alkaline Phosphatase was chosen for this work due to the fact that it is widely expressed in a number of tissues compared to the Intestinal, Placental and Placental-like isoforms, which are tissue specific.<sup>44</sup> Since the 1940s, scientists have been studying the role of AP in hair growth. Increased interest in the role of AP in the hair follicle occurred when dermatopathologists observed decreased or absent AP activity in early alopecia areata, an autoimmune response causing hair loss in patches.<sup>45</sup>

When Johnson and colleagues studied the levels of AP in the Long-Evans hooded rat skin model, they observed slight levels of activity in early anagen. However, they observed a marked increase in AP activity in late anagen, when rapid epithelial proliferation occurs. When the hair follicle is in telogen, AP activity is not observed.<sup>46</sup> In 1952, Hardy and colleagues reported that human hair follicles show an absence of AP in the dermal papilla of telogen follicles.<sup>47</sup> In 1994, Handjiski and colleagues observed AP activity in all stages of hair growth in C57 BL-6 mouse skin. This data conflicts with the previous reports described above. Handjiski and colleagues state that the observed differences might be due to strain specific differences.<sup>45</sup>

Due to the conflicting reports on the presence of AP in the hair follicle only during growth stages, an additional growth control was used in the work reported in this manuscript. Matrix Metalloproteinase-2 (EC 3.4.24.24) has been shown to play a key role in the remodeling and cell migration in the extracellular matrix.<sup>48</sup> Yamazaki and colleagues (1999) observed growth stage dependent expression of MMP-2 mRNA in anterior dorsal skin of female Sprague-Dawley rats. Matrix Metalloproteinase-2 mRNA was strongly expressed in anagen hair follicles and weakly expressed in telogen follicles.<sup>49</sup> By probing all hair follicles with antibodies for both proteins, TNAP and MMP-2, the growth stage of each hair follicle can be determined with good confidence.

Prior to the initiation of the work describe herein, a literature search revealed that almost all published reports using IHC to study the proteins in the hair follicle used sectioned plucked hair follicles or skin biopsies. The IHC was performed using a method similar to that described in this report. Only one other manuscript described the use of unsectioned plucked hair follicles in IHC. Horikowa and colleagues used IHC on the ORS of plucked hair follicles to study DOPA-negative melanocytes.<sup>50</sup> Since the proteins of interest examined in this work were not in the ORS, the method used in this work is slightly different from the method described by Horikowa. By digesting the ORS, the internal structure of the hair follicle bulb was easily examined without time-consuming sectioning.

The modified IHC methodology described in this manuscript was necessary to study the exposure-responsive proteins in intact, plucked rat whisker follicle bulbs. Unlike traditional segmental hair analysis in which the specific chemical or metabolites are extracted from the hair shaft and measured, the methods described in this report can be used to monitor real-time or near real-time changes in the levels of specific follicular bulb biomarkers to determine exposure to toxicants. To date, seventeen OP nerve agent-responsive target proteins have been confirmed as present in the whisker follicle using Western Blotting and IHC. Ongoing studies suggest that this method may be useful in determining exposure to various doses of OP nerve agent VX, as well as to the munitions compound, Royal Demolition Explosive (RDX). Thus this new IHC method and the potential biomarker targets described in this report may be useful in identifying or confirming exposures to a wide variety of military significant toxicants.

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